CONCISE COMMUNICATION

Influence of the incubation atmosphere on the production of biofilm by staphylococci

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Biofilm formation by *Staphylococcus epidermidis*, *Staphylococcus hemolyticus*, *Staphylococcus sciuri* and *Staphylococcus aureus* in aerobic, anaerobic and CO_2 incubation atmospheres was quantified by the modified microtiter plate test. The *S. epidermidis* and *S. aureus* strains showed significantly lower biofilm production when grown in a CO_2-rich environment compared to that exhibited in aerobic incubation. The amount of biofilm produced by these strains under anaerobic conditions did not differ significantly from the biofilm formation detected in the aerobic incubation. The incubation atmosphere did not affect *S. sciuri* biofilm formation. Biofilm production by *S. hemolyticus* isolates was very low regardless of the experimental conditions used.

Keywords Biofilm, staphylococci, atmosphere

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The incidence of foreign body-associated infections caused by staphylococci, particularly coagulase-negative staphylococci, has significantly increased in recent years [1]. It has been proposed that biofilm produced by staphylococci on the surface of in-dwelling medical devices is essential for the pathogenicity of these bacteria [2–5].

The bacterial pathogens must adapt to a wide variety of changing environmental conditions during the infection cycle [6]. Bacteria regulate their gene expression in response to different environmental signals such as temperature, osmolarity, O_2, CO_2, pH, nitrogen compounds, etc. [6]. The influence of incubation atmosphere on biofilm formation by staphylococci has attracted attention [7–11] because it was shown that elevated CO_2 concentrations and variable oxygen levels are encountered in vivo, in contrast to the aerobic conditions predominantly used in laboratory studies. However, contradictory results were obtained in different studies [7–11].

The purpose of the present study was to determine the significance of the incubation atmosphere as a condition for testing biofilm formation in vitro through its effect on biofilm produced by defined staphylococcal species of varying importance in the pathogenesis of foreign body-associated infections.

In total 30 staphylococcal strains were used in this study: four reference strains, *S. aureus* ATCC 25923, *S. aureus* NCTC 8325, *S. epidermidis* ATCC 14990, and *S. sciuri* ATCC 29062; 26 clinical isolates, seven strains of *S. epidermidis*, six strains of *S. hemolyticus*, six strains of *S. aureus*, and seven strains of *S. sciuri*. All nonreference strains were fully identified by the Staphylococcus MIC/ID panel (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) or API Staph (bioMérieux, Marcy-l'Etoile, France). The clinical strains were isolated from various medical devices (central venous catheters, prosthetic heart valves, and drain), except four out of seven *S. sciuri* strains which were isolated from urine.

Stock cultures were maintained on Columbia agar (Torlak, Belgrade, Serbia) supplemented with 5% horse blood at 4 °C. Working cultures were prepared by inoculation on Columbia agar supplemented with 5% horse blood and incubated aerobically at 35 °C for 24 h. The cultures were

used to prepare bacterial suspensions in sterile distilled water adjusted to a 0.5 McFarland standard. The suspensions obtained were inoculated into a brain–heart infusion broth (Becton Dickinson Microbiology Systems) then poured into the wells of plastic microplates.

The modified microtiter plate test [12], with some improvements, was employed for the quantification of biofilm. Wells of sterile 96-well flat-bottomed plastic microplates (Spektar, Čačak, Serbia) were filled with 250 μL of the brain–heart infusion broth. Negative control wells contained the broth only. Twenty μL of bacterial suspension was then added to each well. In order to determine the effect of the atmosphere of incubation on the biofilm production, the inoculated plates were incubated under aerobic conditions, anaerobic conditions obtained with BBL GasPack Anaerobic System Envelopes (Becton Dickinson Microbiology Systems), and in an atmosphere with CO_2 (4%) obtained with ProGas-CO_2 envelopes (Torlak). The plates were incubated at 35 °C for 24 h. Following incubation, the content of each well was aspirated, and each well was washed three times with 300 μL of sterile distilled water. The remaining attached bacteria were fixed with 200 μL of methanol per well, and after 15 min the plates were emptied and left to air dry. Afterwards the plates were stained for 5 min with 160 μL per well of Crystal violet used for Gram staining (Gram-color staining set for microscopy; Merck, Darmstadt, Germany). Excess stain was rinsed off by placing the microplates under running tap water. After the plates were air dried, the dye which was bound to the adherent cells was resolubilized with 160 μL of 33% (v/v) glacial acetic acid (Zorka Pharma, Šabac, Serbia) per well. The optical density (OD) of each well was measured at 570 nm by using an automated Multiskan EX reader (Labsystems, Helsinki, Finland). All tests were carried out three times. The results were averaged and thereafter the differences in the degree of biofilm formation were examined by the Friedman test, followed by the Wilcoxon signed ranks test. *P*-values of < 0.05 were considered significant.

Staphylococci are the predominant cause of foreign body-associated infections. The species tested in the present study were chosen according to the frequencies of their isolation as causative agents of medical device-associated infections. *S. epidermidis* is the most prominent causal organism of these infections. *S. hemolyticus*, a member of the *S. epidermidis* group, is occasionally isolated in infections of implanted medical devices [1]. Although *S. sciuri* has never been reported as the etiological agent of medical device infections, the isolation of this bacterium from central venous catheters has been described [13]. The *S. aureus*, the only coagulase-positive species tested in the study, is generally considered an important pathogen in medical device-associated infections, although the frequency of isolation is not the same as for coagulase negative staphylococci [14].

The effect of the incubation atmosphere on biofilm formation by the tested staphylococcal species is shown in Figure 1. The *S. epidermidis* and *S. aureus* strains showed significantly lower biofilm production when grown in a CO_2-rich environment as compared to the biofilm formation under both aerobic and anaerobic conditions. The amount of biofilm produced by these staphylococci under anaerobic conditions did not differ significantly from the biofilm formation detected in aerobic incubation. The incubation atmosphere did not affect the *S. sciuri* biofilm formation, because there were no significant differences among the levels of biofilm produced, irrespective of the type of incubation atmosphere. Three strains of *S. sciuri* recovered from central venous catheters produced less quantities of biofilm than the strains isolated from urine, but we assume that these three isolates were only contaminants because there was no strong evidence of correlation between the presence of *S. aciuri* strains on central venous catheters and infection in the patients. The overall biofilm production by *S. hemolyticus* isolates was very low regardless of the experimental conditions used; nutritionally very rich medium and different incubation atmosphere.

Previous experiments performed with coagulase-negative staphylococci showed a decreased biofilm production in anaerobic and CO_2 atmospheres, compared to the biofilm formation under aerobic conditions [7–10]. Cramton *et al.* [11] found that their *S. aureus* strain formed biofilm only under anaerobic conditions, while the *S. epidermidis*-tested strain produced biofilm under both aerobic and anaerobic conditions. In addition, the increased CO_2 concentrations (1%) in their experiment did not affect the biofilm production [11].

The results obtained in our study are somewhat different, because a statistically significantly lower amount of biofilm formation was noted only in the

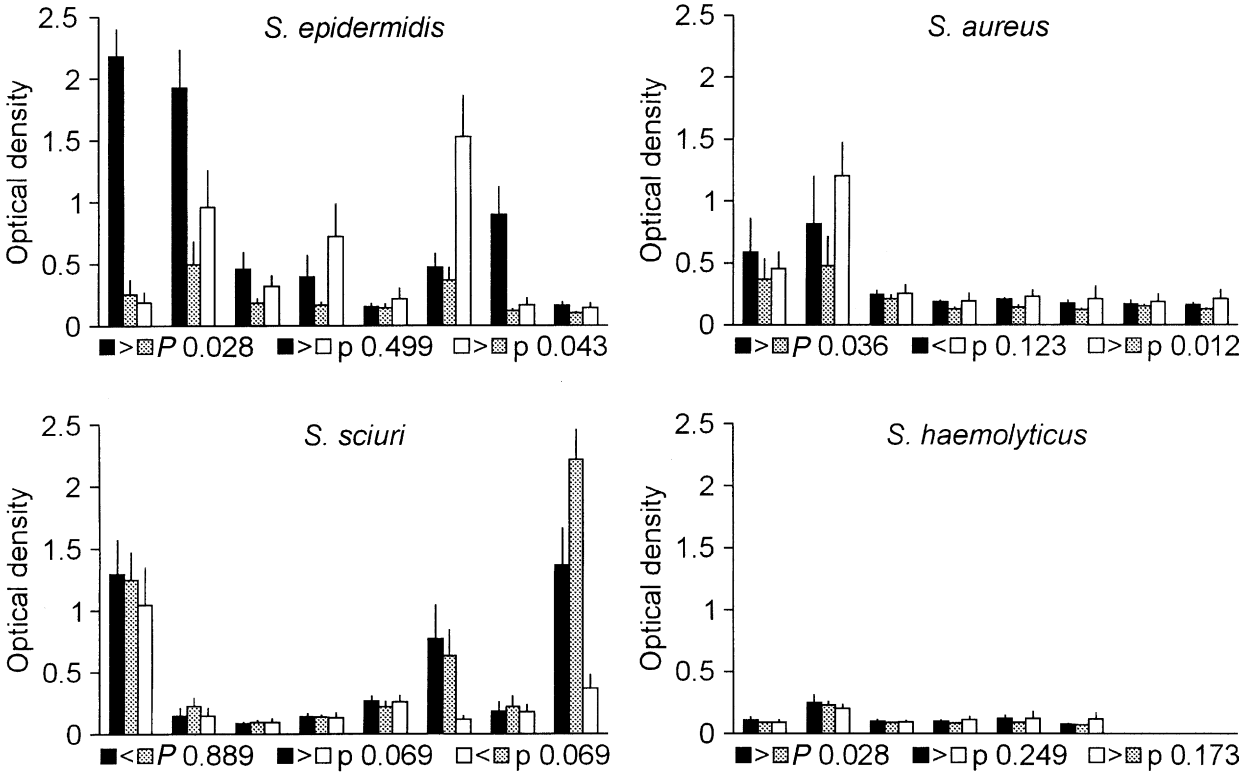

Figure 1 The influence of the incubation atmosphere on the production of biofilm by four *Staphylococcus* species. Bars: the mean optical density (OD) ± SD of the biofilm formed by a particular strain of a species in different gaseous environments (■, aerobic atmosphere; ▨, CO_2; □, anaerobic atmosphere). Boxes and mathematical symbols (< , >): symbols denoting differences among mean OD values of the biofilm produced by all strains of a particular species in different gaseous environments (■, aerobic atmosphere; ▨, CO_2; □, anaerobic atmosphere). The differences were considered as significant if $P < 0.05$.

CO_2-atmosphere incubation. This discrepancy could be explained by the different experimental technique used and/or the differences in the organisms tested. The modified microtiter-plate test, which we used, enabled us to measure bacteria attached both to the bottom as well as to the walls of the wells of a 96-well flat-bottomed plastic tissue culture plates [12], while the most frequently used microtiter plate test of Christensen *et al*. [2] measures only the bacteria attached to the bottom of the wells.

The results obtained in this study show that the capacity for biofilm production in vitro by staphylococcal species known to be causative agents of foreign body-associated infections significantly decreases when these organisms are grown in a CO_2-rich environment. Higher CO_2 tensions are found in most body fluids and tissues. Thus, we recommend that in vitro testing of biofilm formation by these bacteria should include incubation in a CO_2-rich atmosphere. This would provide a more objective and reliable correlation between the in vitro-testing results and biofilm formation by staphylococci in vivo.

REFERENCES

1. Kloos WE, Bannerman TL. Update on clinical significance of coagulase-negative staphylococci. *Clin Microbiol Rev* 1994; 7: 117–40.
2. Christensen GD, Simpson WA, Younger JJ *et al*. Adherence of coagulase-negative staphylococci to plastic tissue culture plates. A quantitative model for the adherence of staphylococci to medical devices. *J Clin Microbiol* 1985; 22: 996–1006.
3. Davenport DS, Massanari RM, Pfaller MA, Bale MJ, Streed SA, Hierholzer WJ. Usefulness of a test for slime production as a marker for clinically significant infections with coagulase-negative staphylococci. *J Infect Dis* 1986; 153: 332–9.
4. Deighton MA, Balkau B. Adherence measured by microtiter assay as a virulence marker for *Staphylococcus epidermidis* infections. *J Clin Microbiol* 1990; 28: 2442–7.

5. Mulder JG, Degener JE. Slime-producing properties of coagulase-negative staphylococci isolated from blood cultures. *Clin Microbiol Infect* 1998; 4: 689–94.
6. Guiney DG. Regulation of bacterial virulence gene expression by the host environment. *J Clin Invest* 1997; 99: 565–9.
7. Barker LP, Simpson WA, Christensen GD. Differential production of slime under aerobic and anaerobic conditions. *J Clin Microbiol* 1990; 28: 2578–9.
8. Denyer SP, Davies MC, Evans JA, *et al.* Influence of carbon dioxide on the surface characteristics and adherence potential of coagulase-negative staphylococci. *J Clin Microbiol* 1990; 28: 1813–7.
9. Hussain M, Wilcox MH, White PJ, Faulkner MK, Spencer RC. Importance of medium and atmosphere type to both slime production and adherence by coagulase-negative staphylococci. *J Hosp Infect* 1992; 20: 173–84.
10. Pérez-Giraldo C, Rodríguez-Benito A, Morán FJ, Hurtado C, Blanco MT, Gómez-García AC. Influence of the incubation atmosphere on the production of slime by *Staphylococcus epidermidis*. *Eur J Clin Microbiol Infect Dis* 1995; 14: 359–62.
11. Cramton SE, Ulrich M, Gotz F, Doring G. Anaerobic conditions induce expression of polysaccharide intercellular adhesin in *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Infect Immun* 2001; 69: 4079–85.
12. Stepanović S, Vuković D, Dakić I, Savić B, Švabić-Vlahović M. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *J Microbiol Meth* 2000; 40: 175–9.
13. Stepanović S, Vuković D, Savić M, Švabić-Vlahović M. *Staphylococcus sciuri*: Recommendation for simple identification. *New Microbiol* 2000; 23: 201–5.
14. Crump JA, Collignon PJ. Intravascular catheter-associated infections. *Eur J Clin Microbiol Infect Dis* 2000; 19: 1–8.